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## CERTIFICATE

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I hereby certify that annexed is a true copy of the Provisional Specification as filed on 17 June 2003 with an application for Letters Patent number 526526 made by MICHAEL GARY NICHOLLS; ERIC ARNOLD ESPINER; TIMOTHY CHARLES RAMSEY PRICKETT; TIMOTHY GRANT YANDLE and ARTHUR MARK RICHARDS.

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Commissioner of Patents, Trade Marks and Designs



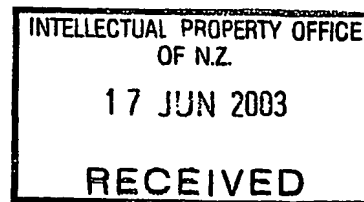
## NEW ZEALAND PATENTS ACT 1953

## PROVISIONAL SPECIFICATION

## NT CNP PEPTIDES AND USES THEREOF

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- 1 -



## **NT-CNP PEPTIDES AND USES THEREOF**

### **FIELD OF THE INVENTION**

This invention relates to amino-terminal CNP peptides and their use in diagnostic and prognostic testing for growth potential, and skeletal development and maturation in mammals including humans. More particularly, the invention relates to the use of these peptides in diagnosis and prognosis of skeletal health, skeletal disorders and disease, and disorders of calcification in mammals, including humans.

### **BACKGROUND TO THE INVENTION**

C-type natriuretic peptide (CNP) is a member of a family of structurally related peptides that play an important role in the control of blood pressure, renal function and volume homeostasis.

CNP has been found in a variety of tissues with significant levels being demonstrated in the brain, pituitary gland, vascular endothelium, bone and cartilage, and kidney. In the vascular system, it has been identified in endothelial cells lining the vasculature where it is believed to play a largely paracrine role in regulating vascular tone and vascular remodelling. Recent evidence suggests that CNP may also be an important regulator of skeletal (including bone and cartilage) growth. Potential applications of plasma or serum CNP assays to detect or monitor vessel and skeletal disease are limited by the low circulating concentrations of CNP, which are close to the detection limit of most analytical methods for this hormone.

CNP assays to detect or monitor vessel and skeletal disease are limited by the low circulating concentrations of CNP, which are close to the detection limit of most analytical methods for this hormone.

ProCNP is synthesised as the 103 amino acid precursor proCNP(1-103) (Figures 1 and 2) which is thought to be subsequently cleaved between amino acid residues 50 and 51 or 81 and 82 to produce the two known biologically active forms of CNP; CNP(51-103) and CNP(82-103) respectively. The latter CNP forms have the trivial names CNP-53 and CNP-22 respectively. Fragments from the amino terminal region of proCNP (NT-CNP) [including proCNP (1-81) and proCNP(1-50)] are present in the circulatory system. It is possible to quantitate circulating NT-CNP peptides and therefore indirectly quantify CNP in tissues and circulation.

Amino terminal CNP fragments (NT-CNP), derived from the aminoterminal region of proCNP(1-103) circulate in humans and other species (1). NT-CNP in humans has substantially higher circulating levels compared to the biologically active forms of CNP. NT-CNP has been partially characterised and measured in haemofiltrates of humans undergoing dialysis (patent WO01/14885). As measured by the method described in that patent, NT-CNP levels in plasma were claimed to be higher in patients with chronic renal disease than in normal subjects. This information has been used to patent a method of measuring NT-CNP with application to the diagnosis and prognosis of endothelial disorders eg arteriosclerosis, and renal failure (patent WO01/14885). Surprisingly, the applicants, using a fully validated assay (1) for NT CNP, have established that Plasma NT CNP levels in patients with arteriosclerosis do not differ from those in normal

subjects. Further, using our assay, any changes in levels of plasma NT CNP found in renal disease are minor when compared to the changes observed during normal growth and development of infants to children and adulthood. . The changes we observe in NT-CNP plasma levels using the validated assay are therefore not related to vascular endothelial function.

Growth of the long bones, initiated by growth hormone and many other hormonal and paracrine factors, is engendered by recruiting chondrocytes from more primitive cells (mesenchymal cells of bone marrow) adjacent to the growth plate of the epiphysis. Linear growth in childhood results from endochondral ossification in the growth plate of long bones and vertebrae. For normal growth to occur there must be a co-ordinated sequence of steps involving (i) proliferation of chondrocytes within the growth plate, (ii) differentiation of chondrocytes to larger cells (hypertrophic phase) which lay down an extracellular matrix, (iii) apoptosis (programmed cell death) and mineralisation of matrix (ossification). The proliferation phase is crucial to the growth process, and is regulated in health largely by paracrine and hormonal factors including growth hormone (GH), IGF-1, thyroxine and glucocorticoids. Growth ceases after pubertal secretion of sex steroids which reduce chondrocyte proliferation and result in epiphyseal fusion.

Little is known of the paracrine factors affecting chondrocyte proliferation. Among other factors is CNP, which acts to stimulate chondrocyte proliferation. Unfortunately, there is virtually no detectable CNP present in circulation as it is effectively sequestered and/or metabolised by cells. However, the measurement of NT-CNP in blood, reflecting the

level in cartilage growth plates, provides for the first time a guide to the volume and function of this tissue.

It is known in the art that a crude estimation of skeletal maturation (and hence potential for further growth) can be determined by a clinician examining an x-ray of the left wrist of a human. This provides an assessment of "bone age" and is a subjective process. Accuracy in measuring bone age is often dependent upon the skill and experience of the clinician. Other markers exist for assessing skeletal growth – eg. alkaline phosphatase, osteocalcin etc. These do not reflect the activity of the remaining cartilage which determines future growth. A need exists for an objective determination of skeletal age in a subject.

It is therefore an object of the present invention to provide a method of detecting levels of NT-CNP for determining skeletal maturation and/or health in the subject; and/or to apply such measurements to the diagnosis and management of disorders of growth and skeletal maturation, and/or to determine the effects of drug and other treatments on skeletal status, or at least to provide the public with a useful choice.

## **SUMMARY OF THE INVENTION**

In one aspect, the present invention provides a method of analysing a sample from a subject to determine skeletal maturation in the subject, comprising the steps of analysing the sample for levels of NT-CNP present in the sample; and comparing the results against a base level of expected NT-CNP levels to determine skeletal maturation in a subject.

In a further aspect, the present invention provides a method of diagnosing skeletal disease in a subject, comprising obtaining a sample of a biological fluid from the subject and determining the levels of NT-CNP in the sample, wherein the levels of NT-CNP are different from a predetermined reference interval for undiseased subjects is an indication of disease.

In a still further aspect, the present invention provides a method of determining levels of NT-CNP which are indicative of skeletal age of the subject, comprising the steps of obtaining a sample of a biological fluid from the subject; analysing the sample for the levels of NT-CNP in the sample; and comparing the levels with results against a base level of expected NT-CNP levels in the subject to determine skeletal age. In one embodiment, the levels determined are expected base line levels in healthy subjects. In another embodiment, the levels determined are base line levels in diseased subjects.

In a yet further aspect, the invention provides a kit comprising a binding agent, an indicator system for signalling the presence of NT-CNP or a fragment thereof when bound to the binding agent, and instructions for analysing the sample to determine whether skeletal disease is present.

In another aspect, the present invention provides a kit comprising antibodies, an indicator system for signalling the presence of NT-CNP or a fragment thereof when bound to the binding agent, and instructions for analysing the sample to determine whether skeletal disease is present.

In another aspect, the present invention provides a method of assessing neonatal health in the newborn, comprising obtaining a sample of a biological fluid from the umbilical cord blood of said subject and determining the level of NT-CNP in the sample, wherein a level of NT-CNP above or below a predetermined reference interval for normal subjects is an indication of poor skeletal health.

In another aspect, the present invention provides a method of assessing neonatal health in the newborn, comprising obtaining a sample of a biological fluid from the umbilical cord blood of said subject and determining the level of NT-CNP in the sample, wherein a level of NT-CNP above or below a predetermined reference interval for normal subjects is an indication of a limited potential for growth.

In another aspect, the present invention provides a use of an NT-CNP ligand and binding agent in the preparation of a diagnostic agent for determining skeletal maturation in a subject.

In another aspect, the present invention provides a use of a NT-CNP ligand and binding agent in the preparation of a diagnostic agent for detecting skeletal disease in a subject.

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In another aspect, the present invention provides a use of a NT-CNP ligand and binding agent in the preparation of a diagnostic agent for determining skeletal health in a subject.



In another aspect, the present invention provides a use of a NT-CNP ligand and binding agent in the preparation of a diagnostic agent for estimating expected skeletal growth in a subject.

In one embodiment, the diagnostic agent is for determining retarded skeletal growth potential in a subject. In another embodiment, the diagnostic agent is for determining increased growth potential in a subject.

In another aspect, the present invention provides a method of determining a change in skeletal status, comprising obtaining a sample of a biological fluid from a subject with a skeletal disorder, either current or incipient, and determining the level of NT-CNP in the sample, and obtaining a later second sample of a biological fluid from said subject and determining the level of NT-CNP in the second sample wherein a change in said levels of NT-CNP is an indication of a change in skeletal status.

In one embodiment, the method determines the effect of drug or other treatments on skeletal health, and monitoring of the skeleton's response to drug and other treatments.

In another embodiment, the method determines the effects of environmental toxins, ionising irradiations and disorders of nutrition on skeletal health, and the monitoring of the skeletons response to correcting interventions.

In another embodiment, the method determines the diagnosis and healing of bone fracture and repair, and the monitoring of the rate of repair to full union of the fractured bone.

In another embodiment, the method determines whether a process of endochondral calcification and ossification within soft tissues outside the skeleton is present.

The aspects above are applicable to mammalian species including humans. Veterinary applications are therefore contemplated within the scope of the invention.

The invention extends to an index produced by the methods of the invention.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** is a scheme showing hypothetical processing of proCNP to CNP-22, CNP-53 and amino terminal CNP fragments.

**Figure 2** shows the published amino acid sequences predicted for proCNP from gene/cDNA sequencing studies. The sequences for bovine, sheep, human, mouse, rat and pig proCNP sequences are aligned. Shaded positions are those that differ from the human sequence.

**Figure 3** Plasma levels of NT-CNP (filled circles) and CNP (open circles) in children (aged 5-16) compared to healthy adult subjects. Mean and standard error of the mean (SEM) are also shown. Plasma NT-CNP levels in 2 children receiving acute chemotherapy and high dose glucocorticoids are also shown.

**Figure 4** is a graph showing the results of a NT-CNP radioimmunoassay. Serial dilutions of normal human plasma (open diamonds), pooled plasma extracts from children (aged 5-

16 years) (open circles), pooled plasma extracts from patients with heart failure (open triangles) demonstrating parallel displacement of  $^{125}\text{I}$  labelled NT-CNP(1-15)Tyr<sup>16</sup> when compared to NT-CNP(1-15) standards (filled circles).

**Figure 5** is a graph showing the results from size exclusion HPLC of immunoreactive NT-CNP (filled circles) and CNP-22 (open circles) in extracts of A) EDTA plasma from normal human subjects, B) EDTA plasma obtained from “pooled” children, C) EDTA plasma from sheep.

**Figure 6** shows the results from size exclusion HPLC of A) pooled maternal plasma and B) matching pooled cord plasma. Column void volume and elution positions or molecular weight markers are shown by arrows. Immunoreactive NT-CNP (closed circles) and CNP (open circles).

**Figure 7** is a graph showing the mean ( $\pm$  SEM) levels of NT-CNP (filled bars) and CNP (open bars) in neonatal umbilical cord plasma compared to maternal levels immediately after delivery.

**Figure 8** is a plot of NT-CNP (filled circles) and CNP (open circles) showing the effect of aging on blood levels in newborn lambs (n=12) versus mature adult sheep (n=11).

**Figure 9:** Effect of dexamethasone in lambs (n=12) on plasma levels of A) NT-CNP and on B) percentage change in alkaline phosphatase. The arrows indicate when the lambs received either dexamethasone (closed circle) or saline (open circle).

## DETAILED DESCRIPTION OF THE INVENTION

In the present invention:

- 1) "Biological fluid" includes plasma, blood, or serum; urine; synovial fluid, cerebrospinal fluid, lymph, seminal fluid, amniotic fluid or any other body fluid.
- 2) Endochondral calcification refers to the process of chondrocyte formation, proliferation, maturation and ossification, whether in or outside the skeleton.
- 3) "Skeletal disorders" Skeletal disorders include but are not limited to the following:
  - a) Disorders affecting the immature skeleton (ie. as found at birth, and/or occurring prior to adulthood).

### i) Congenital disorders –

Osteochondrodysplasias and osteochondrodystrophy including:

- (a) Achondroplasia, hypochondroplasia, achondrogenesis group (type I-IV), spondylodysplasia group (including spondylometaphyseal dysplasia), metatropic dwarfism, short rib dysplasia group (with or without polydactyly), Atelostereogenesis group; Stickler dysplasia group, epiphyseal dysplasia, metaphyseal dysplasia group; dysostosis multiplex group; chondrodysplasia punctata; chondrodysplasia (Blomstrand type); chondrodystrophy group; brachrachia; mesomelic and micromelic dysplasias; bent bone dysplasia group; osteodysplastic primordial dwarfism group; dysplasias with increased bone density; dysplasias with defective mineralizations; Schwartz-Jampel syndrome; dyssegmental

dwarfism; Pelger-Huët anomaly; opsismod dysplasia; Robinow syndrome.

ii) Disorders of skeletal growth and maturation including:

- (a) Delayed development – including prematurity, intrauterine growth retardation, syndrome associated with growth failure (Russell Silver syndrome, Seckel syndrome, Noonan syndrome, progeria Cockayne syndrome, Bloom syndrome, Prader Willi syndrome); placental insufficiency syndromes; maternal subnutrition and/or drug use; infant subnutrition and/or chronic disease; psychosocial deprivation; hypothyroidism; Cushing's syndrome; glucocorticoid treatments and related therapy; pseudohypoparathyroidism; rickets; pituitary growth hormone deficiency; growth hormone insensitivity; Shox mutation; Turner's syndrome; collagen disorders; rheumatoid disorders; constitutional delay of growth and maturation; hypogonadism; delayed puberty; coeliac disease; Crohn's disease; intestinal malabsorption; mitochondrial cytopathies; Down syndrome; chronic anaemias; chronic renal disorders; chemotherapy and cytotoxic drug therapy; irradiation therapy; Vitamin A toxicity or other drug or environmental factors including lead toxins affecting skeletal health

- (b) “Advanced development” syndromes associated with tall stature, including cerebral gigantism, Beckwith-Wiedemann syndrome; gigantism (pituitary growth hormone hypersecretion), sexual precocity, hyperthyroidism, Marfan syndrome, XYY syndrome, homocystinuria, congenital adrenal hyperplasia; Klinefelter’s syndrome, Proteus syndrome, constitutional tall stature, familial tall stature.

b) Disorders affecting the mature skeleton –

- i) All of the above, plus:
- ii) Degenerative disorders of cartilage, including osteoarthritis; rheumatoid arthritis; osteophyte growth; vertebral disc disease.
- iii) Tumours of bone or cartilage – including chondromata, chondroma, chondrofibroma, chondrosarcoma, osteosarcoma, osteoma.
- iv) Bone/cartilage infections, as in osteomyelitis.
- v) Bone/cartilage injury and repair as in fractures, post-surgical (osteotomy) resections etc.
- vi) Bone wasting, as in osteoporosis.
- vii) Disorders requiring transplants of cartilage tissue and their monitoring pre and post-intervention.

c) Other disorders as in –

- i) Endochondral calcification within soft tissues , dystrophic calcification, disorders of calcification including calciphylaxis, fibrodysplasia ossificans

progressiva (FOP), progressive osseous heteroplasia (POH); drug- related skeletal disease, including glucocorticoid use, eg. cytotoxic and chemotherapy; fluorosis; biphosphonates; parathyroid hormone, thyroxine; sex steroids; growth hormone.

- 4) "Skeletal maturation" is the process of skeletal aging to full development when epiphyses ossify, i.e. adult stage.
- 5) "Cartilage maturation" is the process of cartilage aging to full developmental i.e. adult stage.
- 6) "NT-CNP" is amino-terminal pro C-type natriuretic peptide more fully defined below.

In brief, NT-CNP encompasses any discriminatingly detectable sequence of consecutive amino acids selected from the amino acid sequence present in proCNP(1-81). The isolated peptide comprises a partial consecutive sequence of proCNP(1-81) of a suitable size to enable antibodies and other immuno-molecules to be raised against the peptide. Preferably, there should be at least six consecutive amino acids from proCNP(1-81). This provides a sufficient epitope for specific amino acid detection. However, in some cases, it may be necessary to have at least eight amino acids to provide sufficient specificity for the peptide detection process. In a currently preferred embodiment, at least 15 consecutive amino acids from proCNP(1-81) is employed. For the best ability to raise antibodies, proCNP(1-81) can be employed itself. Other examples of peptides that could be used in raising antibodies include proCNP(1-50), proCNP(1-81), and proCNP(51-81) as set forth in Figures 1 and 2. proCNP(1-103) can also be used in certain circumstances as antibodies raised may cross-react with proCNP(1-81) specific antibodies. The peptides useful in the present

invention are collectively referred to as NT-CNP in this application. Variants are preferably functionally equivalent variants.

The amino acid sequence of human proCNP(1-103) and other known mammalian proCNP sequences are shown in Figure 2. From the known and published sequence of the human CNP gene and the inferred amino acid sequence it is readily possible to predict the amino acid sequences of amino terminal CNP peptides that would be produced by correct processing of human proCNP(1-103) to produce the known biologically active peptides CNP(51-103) and CNP(82-103). The main predicted amino terminal CNP peptides are shown in Figure 1 and include proCNP(1-50), proCNP(1-81) and proCNP(51-81), some or all of which may circulate in addition to proCNP(1-103). Further processing or metabolism of these peptides may occur in tissues or in body fluids such that any combination of smaller peptides derived from proCNP(1-50), proCNP(1-81) and proCNP(51-81) and proCNP(1-103) may also circulate. However, the uses of the invention are not limited to the above identified specific N-terminal pro CNP peptides. Any cleaved identifiable non-CNP circulating moiety derived from proCNP(1-103) is useful in the practice of the invention.

The above peptides, their metabolites and subsequences from proCNP(1-81) - a peptide that comprises the aminoterminal end of the precursor peptide proCNP(1-103) - are defined in this application as aminoterminal CNP (NT-CNP). Preferably the NT-CNP peptide comprises a sequence of 8 or more consecutive amino acids selected from proCNP(1-81).



- 7) "Reference interval" is defined as a figure within a statistical band of a representative level, or alternatively a figure with an upper and lower level.
- 8) "Skeletal" includes bone and cartilage and ossification.
- 9) "Subject" includes a foetus, neonate, child, immature and mature (adult) mammals, including humans.
- 10) "Binding agent" is any molecule that binds NT-CNP peptides, including antibodies from any species whether polyclonal or monoclonal, antibody fragments such as Fab and Fab2, humanized antibodies or antibodies modified in other ways including substitution of amino acids, and/or fusion with other peptides or proteins. It also includes receptors or binding proteins from any species or modified forms of them.

The progressive fall in skeletal growth rate (as documented by Tanner and Davies (2)) will be reflected in a steady decline in NT-CNP blood levels (excepting possible changes at puberty). Thus the measurement of NT-CNP in blood will reflect the amount of cartilage remaining in growth plates and will be inversely proportional to the degree of skeletal maturation attained.

Since growth of immature young mammals is dependent upon proliferation and hypertrophy of chondrocytes of long bones it follows that abnormal growth in human infants and children may result from abnormalities of CNP synthesis within growth plates of long bones. As NT-CNP is a by-product of CNP production, it is expected that NT-CNP levels in blood correlates with CNP synthesis within tissues. In growing children, the skeleton is likely to be the main source of CNP synthesis. Other (non-skeletal) sources of NT-CNP include the vascular endothelium, heart tissue, circulating blood

elements and reproductive tissues. The possible contributions of these sources to circulating NT-CNP is likely to be small (with the possible exception of blood cellular elements) since (i) NT-CNP levels are not affected by heart disease (unless very severe), or by severe endothelial disease (as occurs in atherosclerosis)(Table 1); (ii) NT-CNP is not affected by physical stress, eg. surgical procedures (Table 1); (iii) NT-CNP does not show diurnal fluctuation and is uninfluenced by meals (Table 1).

**Table 1.** Plasma NT-CNP concentrations across surgery, meal and selected disease cohorts.

<b>Subjects</b>	<b>Plasma NT-CNP concentrations (pmol/L, mean <math>\pm</math> sem)</b>
<u>Across vascular surgery</u> (n=30)	
Day prior to surgery	33 $\pm$ 3
Day after surgery	30 $\pm$ 3
<u>Across Day</u> (n=6, normal adults)	
0900hr	24 $\pm$ 3
1200hr	23 $\pm$ 2
1300hr (after meal)	23 $\pm$ 2
1500hr	22 $\pm$ 2
<u>Atherosclerosis</u> (n=51) *	33 $\pm$ 3
<u>Pagets Disease</u> (n=2) #	22 $\pm$ 1
<u>Osteomalacia</u> (n=2) #	28 $\pm$ 10
<u>Hyperparathyroidism</u> (n=1)	29

\* Occlusive lower limb arterial disease.

# Increased bone turnover with raised alkaline phosphatase activity.

One aspect of the invention generally provides a measurement of N-terminal CNP in blood that assists in the diagnosis of growth disorders and osteodystrophies, including

chondrodystrophies and dysplasia, that hitherto have been difficult to explain and difficult to categorise and diagnose. Without wishing to be bound by theory, this aspect has important implications for the assessment of children presenting with a growth disorder eg. an abnormal level of NT-CNP outside the age reference range will identify an intrinsic skeletal disorder from the much more common disorder of simple constitutional growth disorder (for example, normal variant short stature or tall stature).

Increased levels of osteoblastic activity in adults (where alkaline phosphatase and osteocalcin are increased, for example Paget's disease of bone, osteomalacia and hyperparathyroidism) are not associated with any change in blood NT-CNP level (Table 1). This further supports the specificity of NT-CNP assays as a marker of cartilage as opposed to osteoblastic proliferation.

The present invention also provides a method for diagnosing disorders of growth and skeletal maturation, and skeletal disease. As shown in the examples below, children have a statistically significant higher level of NT-CNP in circulation compared to healthy adult subjects. Therefore, an indicating system that detects levels that deviate from normal is preferred. The indicating system may also be applied in combination with other biochemical analytes, hormone levels, and x-rays etc to improve diagnosis or prognosis.

The radioimmunoassay employing antiserum raised against proCNP(1-15) was used to measure the concentration of NT-CNP in human plasma. Data from twenty one children and sixteen healthy adult subjects showed that NT-CNP levels were significantly raised in plasma from young growing subjects compared to normal adult subjects (Figure 3).

The levels measured in normal healthy subjects (adults as well as children) are much higher than CNP-22 levels in the same sample. NT-CNP levels will be higher than their matching circulating CNP levels. Thus NT-CNP provides a better discriminator because it is more easily detectable in circulation than CNP.

It has been found by the Applicants that NT-CNP fragments produced during processing of proCNP(1-103) are useful markers for the presence of processed active forms of CNP activity in tissues and fluids. Accordingly, any NT-CNP peptide provides practical markers for CNP levels/activity in tissue and in the skeleton. It is preferred that the fragment comprise NT-CNP(1-50), which the Applicants have demonstrated is a major NT-CNP peptide circulating in humans, or its metabolites of shorter length. proCNP(1-81) and proCNP(51-81) and their metabolites are also useful. These are predicted to arise and circulate as indicated in Figure 1. NT-CNP levels reflect CNP levels in tissues and fluids. However, NT-CNP levels may be used on their own without reference to CNP levels.

#### **NT-CNP measurement in body fluids**

Antisera specific to other epitopes in the NT-CNP(1-81) sequence can be produced by the same method as that outlined below in the Examples section by substituting any peptide sequence of eight or more amino acids from the NT-CNP(1-81) amino acid sequence for the NT-CNP(1-15) peptide provided in the example.

**Other methods for NT-CNP measurement**

As stated above, NT-CNP comprises a number of peptide fragments. These fragments may be quantitated in blood or plasma by immunoassays such as ELISA or RIA using antibodies of the invention.

Immunoassays specific for NT-CNP peptides require the production of antibodies that specifically bind to NT-CNP peptides. One such preferred antibody recognises amino acids within proCNP(1-15). These antibodies, while being specific for NT-CNP peptides have broad NT-CNP specificity. Antibodies of the invention preferably bind to one or more of the four peptides proCNP(1-50), proCNP(1-81), proCNP(51-81) and proCNP(1-103) (Figures 1 and 2) or their metabolites. The antibodies can be used to construct immunoassays with broad specificity, as in competitive binding assays below, or used in conjunction with other antibodies described below in sandwich type assays to produce assays specific to each of the three peptides or to other NT-CNP peptides. The latter antibodies for sandwich immunoassays include those specific for amino acid sequences within proCNP(1-15), proCNP(36-50) and proCNP(67-81). These are preferred antibodies of the invention.

In one embodiment, the methods are performed using a kit comprising a binding agent of the invention and an indicator system for indicating the presence of NT-CNP when bound to the binding agent. Such systems are employed in ELISA and RIA methods, which may be used herein.

Binding agents with specificity for NT-CNP and fragments thereof are useful in methods for determining the presence of NT-CNP. Preferably the binding agent has low cross-reactivity with other mediators of growth and skeletal maturation. These binding agents may include antibodies or fragments thereof such as Fab, and F(ab)<sub>2</sub>, prepared using NT-CNP intact polypeptides or fragments thereof as immunising antigens. The antigen may also be coupled to a carrier as desired. Preferred binding agents are antibodies. These include monoclonal antibodies and polyclonal antibodies. These binding agents may be produced by any number of techniques available in the art. For reasons of specificity, monoclonal antibodies are currently preferred. It will be appreciated that humanised antibodies are not required for *in vitro* assays.

Methods for raising antibodies and constructing immunoassays are common knowledge.

Monoclonal antibodies may be produced by known art methods. These include the immunological method described by Kohler et al (3) as well as the recombination DNA method described by Huse et al (4). The use of recombinant phage antibody systems to produce single chain variable antibody fragments, and subsequent mutation (such as site specific mutagenesis) or chain shifting to produce antibodies to NT-CNP peptides is also contemplated.

For example, the host described above may be sacrificed and its spleen removed. The messenger RNA (mRNA) are then isolated and cDNA made from the mRNA using specific primers for the heavy and light chains of the variable region of the antibodies and the polymerase chain reaction (PCR) amplification. The DNA sequences for the heavy

and light chains are joined with a linker sequence, to ensure the correct reading frame. Then the DNA construct will be inserted into a vector, for example, a plasmid or bacteriophage, or virus, for transformation into a host. A preferred vector is a bacteriophage.

Suitable hosts may be selected from prokaryotic, yeast, insect or mammalian cells. Preferably, a prokaryotic host, and most preferably *Escherichia coli* is used. The bacteriophage produces a viral coat and the antibody fragments are expressed on the coat, a phage display library. The phage display library can be screened for antibody fragments with the appropriate affinity for the specific antigens. The library can be screened many times and modifications can be made to the antibody construct through protein engineering techniques, such as site directed mutagenesis and chain shuffling all of which are within the capabilities of the art skilled worker.

The antibodies useful in the invention are particularly useful in immunoassays for determining the presence and/or amount of isolated peptides of the invention in a sample. Sample materials include cells, cell membranes and biological fluids but are not limited thereto. In terms of the present invention, usually a biological fluid selected from blood, plasma, serum or urine. The sample is tested *in vitro* or *in vivo*.

Conventional procedures for generating polyclonal antibodies are detailed in Harlow and Lane (5). Briefly, the protocol requires immunisation of a selected animal host such as a rabbit, goat, donkey, sheep, rat or mouse (usually a rabbit), with an isolated peptide of the invention on a number of spaced occasions, with one or more test bleeds preceding

exsanguination and blood collection. Serum may be separated from clotted blood by centrifugation. Serum may be tested for the presence of polyclonal antibodies using ELISA or radioimmunoassay competitive assays or art equivalent methods.

Antibodies specific to proCNP(1-15), proCNP(36-50) and proCNP(67-81) can be raised after first conjugating these or similar peptides to a large protein such as bovine serum albumin or bovine thyroglobulin to make them immunogenic. Coupling can be effected by use of any protein crosslinking agent including for example the common agents glutaraldehyde, carbodiimide or N-(ε-maleimido-caproyloxy) succinimide ester (MCS) - providing a cysteine residue is added to the peptide sequence prior to coupling. Injection of these conjugates into rabbits, sheep or other species at monthly intervals followed by collection of blood samples two weeks later will enable production of polyclonal antibodies.

Polyclonal and monoclonal antibodies can be used in competitive binding or sandwich type assays. The Applicants provide a competitive binding assay (radioimmunoassay format) in the example section of this application. In this method the liquid sample is contacted with the antibody and simultaneously or sequentially contacted with a labelled NT-CNP peptide or modified peptide containing the epitope recognised by the antibody.

The label can be a radioactive component such as  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$  or a nonradioactive component that can be measured by time resolved fluorescence, fluorescence, fluorescence polarisation, luminescence, chemiluminescence or colorimetric methods. These compounds include europium or other actinide elements, acridinium esters,



fluorescein, or radioactive material such as those above, that can be directly measured by radioactive counting, measuring luminescent or fluorescent light output, light absorbance etc. The label can also be any component that can be indirectly measured such as biotin, digoxin, or enzymes such as horseradish peroxidase, alkaline phosphatase. These labels can be indirectly measured in a multitude of ways. Horseradish peroxidase for example can be incubated with substrates such as o-Phenylenediamine Dihydrochloride (OPD) and peroxide to generate a coloured product whose absorbance can be measured, or with luminol and peroxide to give chemiluminescent light which can be measured in a luminometer. Biotin or digoxin can be reacted with binding agents that bind strongly to them; eg avidin will bind strongly to biotin. These binding agents can in turn be covalently bound or linked to measurable labels such as horseradish peroxidase or other directly or indirectly measured labels as above. These labels and those above may be attached to the peptide or protein: - during synthesis, by direct reaction with the label, or through the use of commonly available crosslinking agents such as MCS and carbodiimide, or by addition of chelating agents.

Following contact with the antibody, usually for 18 to 25 hours at 4°C, or 1 to 240 minutes at 30°C to 40°C, the labelled peptide bound to the binding agent (antibody) is separated from the unbound labelled peptide. In solution phase assays, as in the example provided, the separation may be accomplished by addition of an anti gamma globulin antibody (second-antibody) coupled to solid phase particles such as cellulose, or magnetic material. The second-antibody is raised in a different species to that used for the primary antibody and binds the primary antibody. All primary antibodies are therefore bound to the solid phase via the second antibody. This complex is removed from solution

by centrifugation or magnetic attraction and the bound labelled peptide measured using the label bound to it. Other options for separating bound from free label include formation of immune complexes, which precipitate from solution, precipitation of the antibodies by polyethyleneglycol or binding free labelled peptide to charcoal and removal from solution by centrifugation or filtration. The label in the separated bound or free phase is measured by an appropriate method such as those presented above.

Competitive binding assays can also be configured as solid phase assays that are easier to perform and are therefore preferable to those above. This type of assay uses plates with wells (commonly known as ELISA or immunoassay plates), solid beads or the surfaces of tubes. The primary antibody is either adsorbed or covalently bound to the surface of the plate, bead or tube, or is bound indirectly through a second anti gamma globulin or anti Fc region antibody adsorbed or covalently bound to the plate. Sample and labelled peptide (as above) are added to the plate either together or sequentially and incubated under conditions allowing competition for antibody binding between NT-CNP in the sample and the labelled peptide. Unbound labelled peptide can subsequently be aspirated off and the plate rinsed leaving the antibody bound labelled peptide attached to the plate. The labelled peptide can then be measured using techniques described above.

Sandwich type assays are more preferred for reasons of specificity, speed and greater measuring range. In this type of assay an excess of the primary antibody to NT-CNP is attached to the well of an ELISA plate, bead or tube via adsorption, covalent coupling, or an anti Fc or gamma globulin antibody, as described above for solid phase competition binding assays. Sample fluid or extract is contacted with the antibody attached to the

solid phase. Because the antibody is in excess this binding reaction is usually rapid. A second antibody to NT-CNP is also incubated with the sample either simultaneously or sequentially with the primary antibody. This second antibody is chosen to bind to a site on NT-CNP that is different from the binding site of the primary antibody. These two antibody reactions result in a sandwich with the NT-CNP from the sample sandwiched between the two antibodies. The second antibody is usually labelled with a readily measurable compound as detailed above for competitive binding assays. Alternatively a labelled third antibody which binds specifically to the second antibody may be contacted with the sample. After washing the unbound material the bound labelled antibody can be measured by methods outlined for competitive binding assays. After washing away the unbound labelled antibody, the bound label can be quantified as outlined for competitive binding assays.

A dipstick type assay may also be used. These assays are well known in the art. They may for example, employ small particles such as gold or coloured latex particles with specific antibodies attached. The liquid sample to be measured may be added to one end of a membrane or paper strip preloaded with the particles and allowed to migrate along the strip. Binding of the antigen in the sample to the particles modifies the ability of the particles to bind to trapping sites, which contain binding agents for the particles such as antigens or antibodies, further along the strip. Accumulation of the coloured particles at these sites results in colour development are dependent on the concentration of competing antigen in the sample. Other dipstick methods may employ antibodies covalently bound to paper or membrane strips to trap antigen in the sample. Subsequent reactions employing second antibodies coupled to enzymes such as horse radish peroxidase and

incubation with substrates to produce colour, fluorescent or chemiluminescent light output will enable quantitation of antigen in the sample.

In one embodiment, an antibody with specificity to ProCNP(1-15) is used.

### **Applications of NT-CNP in skeletal physiology and skeletal disorders**

Particular preferred uses of this aspect of the invention permit:

1. The development of NT-CNP in blood as an index of growth rate/maturity and skeletal age. "Skeletal age" represents the degree to which epiphyseal centres in long bones are ossified. In contrast, the level of NT-CNP in blood reflects the amount of cartilage remaining in the growth plates and therefore available for proliferation, extension of long bones and allowing increases in linear growth. Thus, the level of NT-CNP in blood provides an index of future growth potential not previously available. From knowledge of the normal reference range (currently being compiled in children and maturing adults), indexed to known parameters (gender, chronological age, absolute height, bone age and growth velocity) – all themselves referenced to the growth curves of normal children – see Tanner et al (2), the NT-CNP measurement provides the clinician with an objective assessment of the subject's future growth potential. Repeated measurements of NT-CNP in blood at specified intervals provides additional information on growth trends. This approach provides a means of monitoring and assessing efficacy of therapeutic interventions.
2. Detection of reduced or inappropriate levels (ie. levels outside the expected reference range when the child's clinical status is taken into account) of NT-CNP will be found in children or maturing adults with achondroplasia, hypochondroplasia and related

disorders. "Inappropriate" levels also allow for the possibility that levels of NT-CNP may be increased, compared to the reference range – for example, in conditions where CNP is synthesised normally but is unable to exert an effect on cartilage because of a block further down the path which normally leads to cartilage proliferation.

3. Detection of reduced or inappropriate levels of NT-CNP in a subgroup of children with normal variant growth failure (also known as constitutional short stature).
4. Detection of increased or inappropriate levels of NT-CNP, compared to healthy normal children, will be found in some children with accelerated growth, familial tall stature and some other conditions associated with tall stature including Klinefelter's syndrome, Marfan's syndrome, XYY karyotype and premature puberty.
5. Detection of increased or inappropriate levels of NT-CNP in infants with skeletal dysplasia including a disorder known as fibrodysplasia ossificans progressiva (FOP).
6. Discernment between infant skeletal dysplasia and progressive osseous heteroplasia (POH) which chiefly affects ossification within membranous bone rather than endochondral bone. As stated, infant skeletal dysplasia is characterised by elevated or inappropriate NT-CNP levels whereas progressive osseous heteroplasia is not.
7. Detection of decreased or inappropriate levels of NT-CNP in congenital skeletal disorders and other osteodystrophies and chondrodysplasias.
8. Detection of poor growth potential in the newborn, in prematurity, intrauterine growth retardation and disorders of placental function.

Other applications of the assay will include adult and childhood subjects with abnormal skeletal formation including:

1. Detecting abnormal NT-CNP levels in biological fluid (including synovial fluid from joint aspiration) in subjects with loss of cartilage, as in osteoarthritis. "Abnormal" denotes a level outside the normal reference range for the subject's age and gender given regard to clinical status. Since an early step in the development of osteoarthritis is cartilage proliferation, sequential changes in NT-CNP provide information important in diagnosis and treatment.
2. Detecting abnormal NT-CNP levels in biological fluid (including synovial fluid from joint aspiration) in subjects with inflammatory skeletal conditions, including rheumatoid arthritis.
3. Detecting pathological states in which endochondral calcification occurs in soft tissues outside the skeleton
4. Primary neoplastic skeletal states including chondromata, chondrosarcoma, osteogenic sarcoma and related disorders.

The finding of an abnormal level of NT-CNP and by implication a disorder of CNP synthesis or action in bone is important *inter alia* because it focuses diagnostic attention on cartilage growth and avoids multiple other lines of diagnostic testing evaluating the larger number of other disorders affecting growth in children.

Statistical analysis of reference range results obtained in the aspects above is possible using conventional statistical techniques in the art. Preferably, the statistical grouping should include 95% of the sample, more preferably 99% of the sample.

The invention will be described below with reference to the Examples, which are intended to illustrate the invention but are not intended to limit the scope of the invention.

## EXAMPLES

Peptides: Human NT-CNP(1-15), human NT-CNP(1-15)-Tyr<sup>16</sup> and human NT-CNP(1-19) were synthesised by Mimotopes, Australia. Purity by mass spectrometry was 74%, 80% and 95% respectively.

Conjugation and immunization: Human NT-CNP(1-15) (5mg) was conjugated to 4.2mg of bovine serum albumin (BSA, fraction V, GibcoBRL, Life Technologies) using 62.2mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide at pH 7. Rabbits were given a primary subcutaneous injection of the antigen in complete Freund's adjuvant (Sigma Chemical Co., St Louis, MO, USA) and subsequent injections in incomplete Freund's at monthly intervals. Rabbits were bled two weeks after injection.

RIA for N-terminal CNP: NT-CNP(1-15)-Tyr<sup>16</sup> (5µg) was iodinated using 0.5mCi Na<sup>125</sup>I in the presence of 10µg chloramine-T in 5µl 0.5M phosphate buffer, pH 7.5 for 30 seconds, followed by the addition of 50µg cysteine, 25µg BSA and 20µg KI in 100µl buffer. The resulting mixture was loaded onto a 10cm RP300 Brownlee column and eluted with a gradient from 0-60% acetonitrile in 49mM KH<sub>2</sub>PO<sub>4</sub> pH 2.9 over 30 min at 1ml/min, collecting 0.5ml fractions. The fraction containing the major peak of radioactive NT-CNP(1-15)-[<sup>125</sup>I]Tyr<sup>16</sup> was used in the RIA. Peptide standards were made from synthetic human NT-CNP(1-19) taking into account the purity data supplied. All standards, sample extracts, antisera and tracer solutions were made up in assay buffer consisting of 0.1% bovine serum albumin, 0.01% sodium azide, 0.1% triton X100, and 0.05% sodium chloride in 0.1M phosphate buffer pH 7.4. 200µl of sample extract or 7-

38,000 pM NT-CNP(1-19) standard (all in duplicate) were pre-incubated with 100 $\mu$ l antiserum solution at a dilution of 1:6000 for 22h at 4°C prior to the addition of 100 $\mu$ l tracer solution (NT-CNP(1-15)-[<sup>125</sup>I]Tyr<sup>16</sup>) containing 2000 cpm for a further 24h at 4°C. Bound and free NT-CNP(1-15)-[<sup>125</sup>I]Tyr<sup>16</sup> were separated by a solid phase second antibody method (Sac-cell, Donkey-Anti Rabbit, IDS Ltd, England).

RIA for CNP-22: Peptide standards were made from synthetic human CNP-22 (Peninsula Laboratories, Inc. Belmont, California , USA). All standards, sample extracts, antisera and tracer solutions were made up in assay buffer consisting of 0.1% bovine serum albumin, 0.01% sodium azide, 0.1% triton X100, and 0.05% sodium chloride in 0.1M phosphate buffer pH 7.4. 100 $\mu$ l of sample extract or 3-200pM CNP-22 standard (all in duplicate) were pre-incubated with 100 $\mu$ l rabbit anti-C-Type Natriuretic Peptide-22 serum (Peninsula Laboratories, Inc. Belmont, California , USA) for 22 h prior to the addition of 8000cpm [<sup>125</sup>I] labelled [Tyr<sup>0</sup>]-CNP (Peninsula Laboratories, Inc. Belmont, California , USA) and incubated for a further 24 h at 4°C. Bound and free [<sup>125</sup>I] labelled [Tyr<sup>0</sup>]-CNP-22 were separated by a solid phase second antibody method (Sac-cell, Donkey-Anti Rabbit, IDS Ltd, England).

Extraction of peptides from plasma: Blood was collected into tubes containing EDTA, centrifuged and the plasma stored at -80°C. Plasma or urine samples (4-5ml) were extracted using Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts, USA) prewashed with 5mL methanol and 5mL 0.1% trifluoroacetic acid. Plasma or urine was run slowly through the column, which was then washed with 5mL 0.1% trifluoroacetic acid. NT-CNP was then eluted with 80% isopropanol in 0.1% trifluoroacetic acid and dried under an air stream at 37°C after addition of 10 $\mu$ L of 1% triton X100. Extracts were re-



suspended in either 0.1% TFA or 20% CH<sub>3</sub>CN in 0.1% TFA prior to size exclusion or reverse phase HPLC.

## Results

Specificity of the NT-CNP radioimmunoassay: We have established a radioimmunoassay for NT-CNP measurement using an antiserum (J39) raised in rabbits to a synthetic peptide corresponding to the first 15 amino acids of human ProCNP(1-103). A typical standard curve and parallel dilution curves of human plasma is shown in Figure 4. This assay had an ED<sub>50</sub> of 110pM and a detection limit (2 SD from zero) of 2pM. Cross reactivities using the J39 antisera were: - hANP(99-126) <0.03%, hBNP32 <0.05%, hCNP-22 <0.03%, hCNP-53 <0.07%, hproANP(1-30) <0.07%, hproBNP(1-21) <0.4%.

Human and ovine plasma contain NT-CNP: Plasma obtained from human umbilical cords, normal adults, children and sheep was extracted on Sep Pak columns and subjected to size exclusion HPLC (SE-HPLC) (Figures 5 and 6). SE-HPLC showed the major immunoreactive NT-CNP peak in all these samples had a molecular weight close to 5 kDa (fractions 29-30, Figures 5 and 6). No immunoreactivity to antisera raised against CNP-22 could be demonstrated in these HPLC fractions.

These results show the presence of N-terminal CNP fragment(s) in human and sheep plasma. The NT-CNP radioimmunoassay is specific for the pro-CNP(1-15) region of proCNP(1-103) and has less than 0.4% crossreactivity with CNP-22, CNP-53 and other peptides including peptide sequences from other sections of proCNP(1-103). The radioimmunoassay specificity established that the major immunoreactive NT-CNP component in human and sheep plasma contained at least some portion of the proCNP(1-

15) sequence and in conjunction with size exclusion HPLC showed the major NT-CNP component had a molecular weight of 5kDa. A minor NT-CNP component with Mr of 9 kDa was also present (1). Taken together, the data show the new NT-CNP peptides are either proCNP(1-50) (predicted Mr 5046) and proCNP(1-81) (predicted Mr 8.7 kDa) or closely similar peptides. These peptides are products expected from processing of proCNP(1-103) to proCNP(51-103) ie (CNP-53) and to proCNP(82-103) (CNP-22) (Figure 1).

Plasma NT-CNP levels in normal adults and children: The mean  $\pm$  SEM plasma levels of NT-CNP in normal adults ( $21 \pm 4$  pmol/L) are significantly higher than CNP-22 ( $0.8 \pm 0.2$  pmol/L;  $p < 0.001$ ), Figure 7. In children, plasma levels of NT-CNP ( $48 \pm 2$  pmol/L) are significantly higher than in normal adults ( $21 \pm 4$  pmol/L), and much higher than CNP-22 ( $1 \pm 0.05$  pmol/L). Similarly, the massively raised NT-CNP levels found in umbilical cord blood (representing neonatal levels, mean  $246 \pm 17$  pmol/L,  $n=10$ ) are some 60-fold higher than CNP-22 levels measured in the same blood sample (Figure 7).

Children receiving chemotherapy and glucocorticoids for cancer-related diseases have extremely low levels of NT-CNP (Figure 3), indicating inhibition of cartilage proliferation and harmful effects on skeletal health and growth potential. Monitoring the level of NT-CNP in blood or other biological fluid will help in the choice of type of drug therapy and dosage.

Plasma NT-CNP levels in lambs and sheep:

Healthy young sheep (6 months of age) have levels of NT-CNP similar to adult humans (20-34pmol/L). In 12 growing lambs plasma levels of NT-CNP were  $62 \pm 2$  pmol/L at 1 week of age and fell progressively over the following 26 weeks to  $27 \pm 1$  pmol/L ( $p < 0.0001$ ). Levels of CNP-22 in these same sheep were  $5.9 \pm 0.5$  pmol/L (1 week) and  $1.4 \pm 0.1$  pmol/L at 27 weeks Figure 8. The progressive fall of NT-CNP in lambs is consistent with the progressive reduction in cartilage tissue in long bones as the animal matures.

Acute administration of the glucocorticoid dexamethasone, significantly reduces the blood level of NT-CNP, as well as alkaline phosphatase (a marker of mature chondrocyte population), in growing lambs (Figure 9). These results are consistent with prior art showing deleterious effects of corticoids on chondrocytes and growth in children. The use of NT-CNP assays provides a totally novel and easy means of monitoring this effect.

The identification of differences in levels of circulating NT-CNP in various individuals for the first time allows for the provision of various methods and uses of the invention.

## **INDUSTRIAL APPLICATION**

The uses of NT-CNP and binding agents thereof in assays for the prognosis and diagnosis of disorders of growth and skeletal maturation are of industrial significance. In particular, the use of NT-CNP assays in combination with measurements of other growth factors, hormones and/or plasma analytes, may be of particular significance in the healthcare industry, veterinary practice and animal husbandry

The antibodies described herein are useful in immunoassays such as the well known Enzyme Linked Immunosorbent Assays (ELISA) or radioimmunoassays (RIA). These assays may be used to measure the concentration of NT-CNP in plasma or blood. The measured NT-CNP concentrations can then be applied to the diagnosis of conditions or diseases associated with abnormal levels of NT-CNP peptides in circulation. NT-CNP measurements may also be used to monitor conditions in subjects, to guide their treatment and to monitor their response to it.

In combination with x-rays etc, NT-CNP testing could also provide new improved methods to determine skeletal maturity and to diagnose subjects with insufficient or excessive skeletal growth rates.

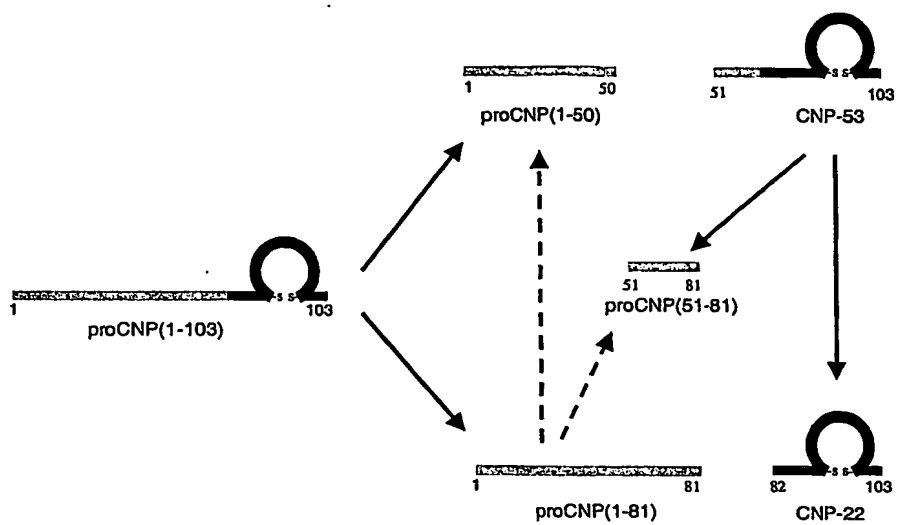
In combination with other tests, NT-CNP testing (in biological fluid such as synovial fluid and/or tissues) could also provide diagnostic information in other skeletal disorders including, but not limited to, disorders of cartilage formation (eg. tumours of the skeleton – chondromas, chondrosarcoma, osteogenic sarcoma), loss of cartilage (osteoarthritis), proliferation of cartilage (as in rheumatoid arthritis). NT-CNP testing will also be helpful in the management of fractures and in assessing rate of healing (fracture repair and bone union).

The assays will also have application in research studies where skeletal health and development is the subject of interest, both in humans and experimental animals or tissues.

Those skilled in the art will of course appreciate that the above description is provided by way of example only and that the invention is not limited thereto.

The following references are hereby incorporated in their entirety by reference:

1. Prickett TC, Yandle TG, Nicholls MG, Espiner EA, Richards AM. Identification of amino-terminal pro-c-type natriuretic peptide in human plasma. *Biochemical and Biophysical Research Communications* 2001;286(3):513-517.
2. Tanner JM, Davies PS. Clinical longitudinal standards for height and height velocity for North American children.[comment]. *Journal of Pediatrics*. 1985;107(3):317-29.
3. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256(5517):495-7.
4. Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 1989;246(4935):1275-81.
5. Harlow E, Lane D. *Antibodies: a laboratory manual*. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory; 1988.



Hypothetical processing pathways of proCNP

Figure 1

**Bovine** KPGAPPKVPRTSGEEVAEPQAAAGGQKGGDKIPGGGGANLKQDRSRLRLDLRVDTKSRRAAWRLLEHHPNARKYGGNNKGLSKGCGFLKLDLRIGSMGSLGC

**Sheep** KPGAPPKVPRTPPGEEVAEPQAAAGGQKGGDKIPGGGGANLKQDRSRLRLDLRVDTKSRRAAWRLLEHHPNARKYGGNNKGLSKGCGFLKLDLRIGSMGSLGC

**Human** KPGAPPKVPRTPPAELEAEPQAAAGGQKGGDKAPGGGGANLKGDERSRLRLDLRVDTKSRRAAWRLLEHHPNARKYKGANKKGLSKGCGFLKLDLRIGSMGSLGC

**Mouse** KPGPPPKVPRTPPGEELADISQAAAGGQKGGDKIPGGGGANLKGDERSRLRLDLRVDTKSRRAAWRLLEHHPNARKYGGNNKGLSKGCGFLKLDLRIGSMGSLGC

**Rat** KPGPPPKVPRTPPGEELAEPAAGGQKGGDKIPGGGGANLKGDERSRLRLDLRVDTKSRRAAWRLLEHHPNARKYGGNNKGLSKGCGFLKLDLRIGSMGSLGC

**Pig** KPGAPPKVPRTPPGEEVAEPQAAAGGQKGGDKIPGGGGANLKGDERSRLRLDLRVDTKSRRAAWRLLEHHPNARKYGGNNKGLSKGCGFLKLDLRIGSMGSLGC

proCNP → CNP-53 → CNP-22

1 10 20 30 40 50 60 70 80 90 100

Figure 2

Fig 2/9





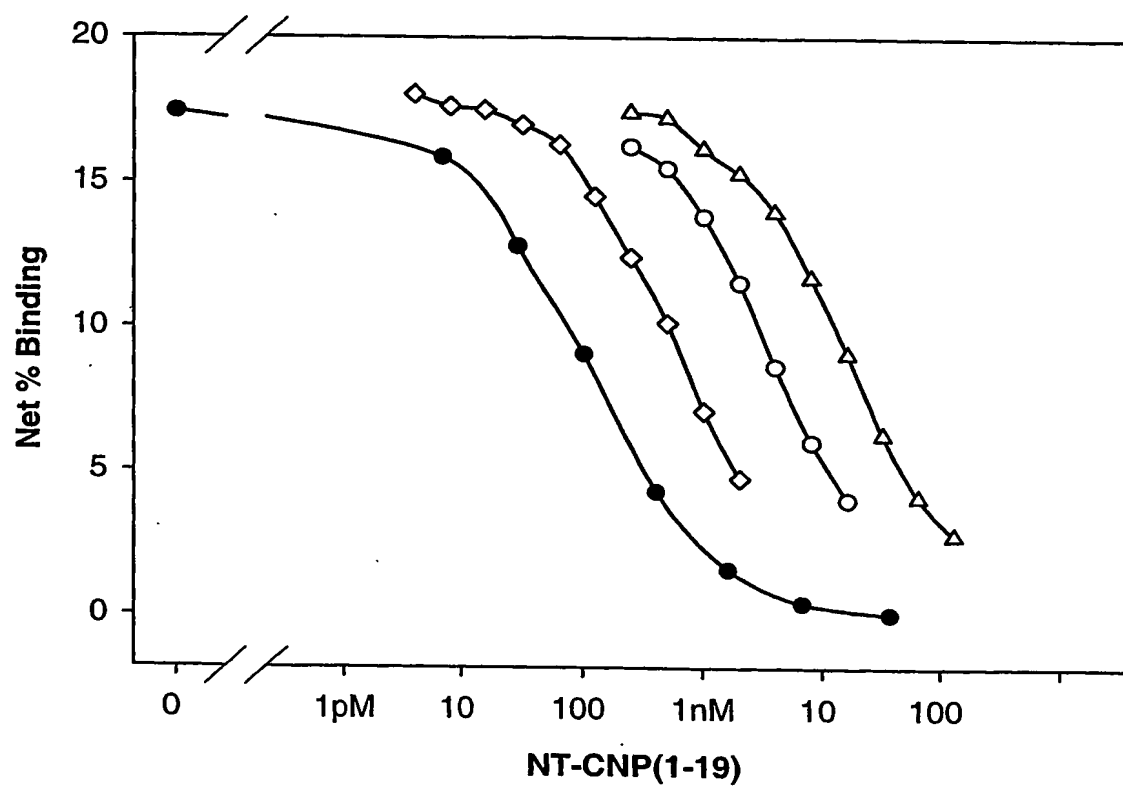
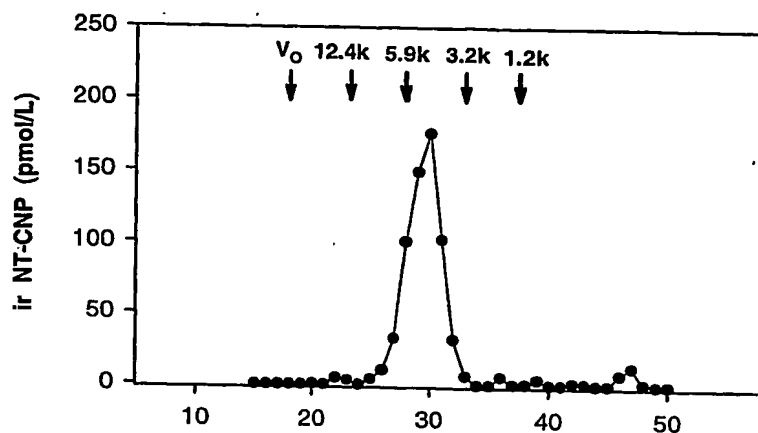
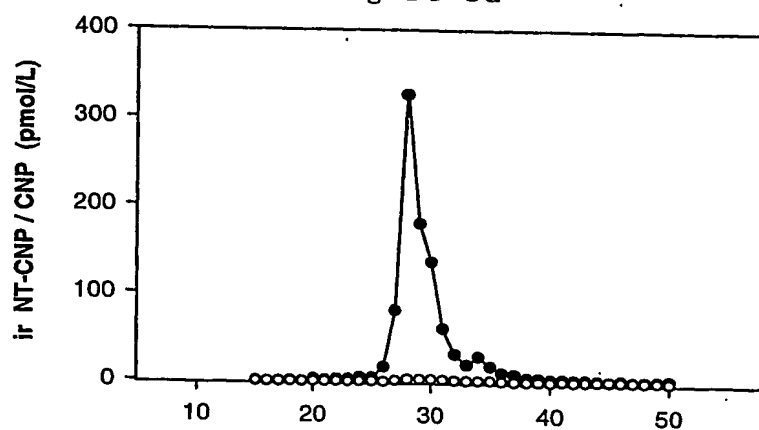


Figure 4



**B**

Figure 5a



**C**

Figure 5b

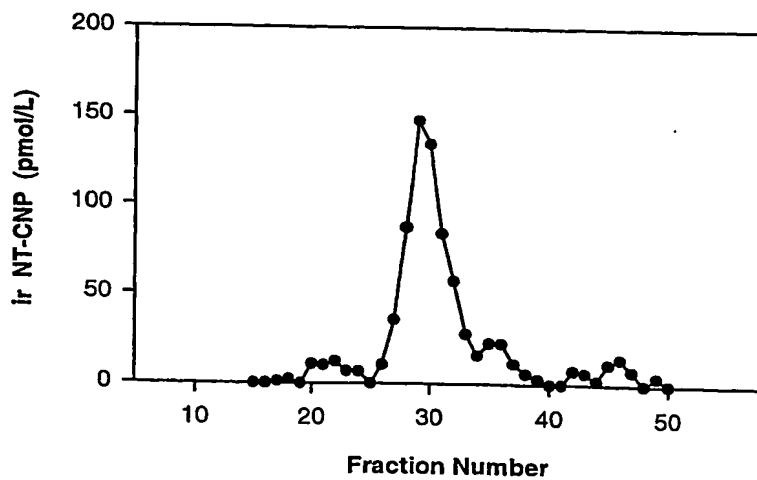


Figure 5c

**A**

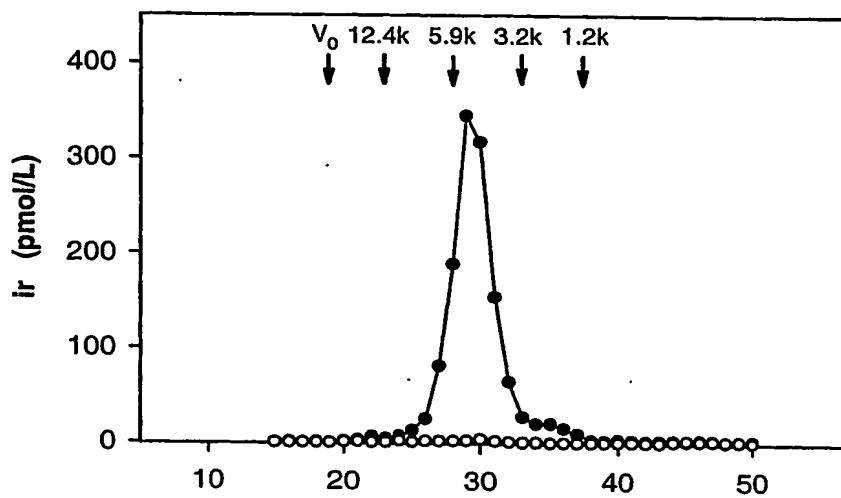


Figure 6a

**B**

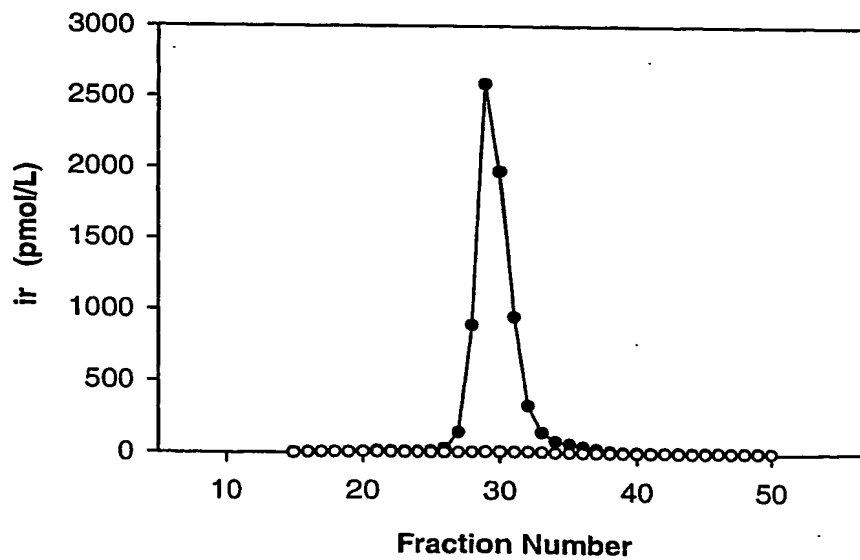


Figure 6b

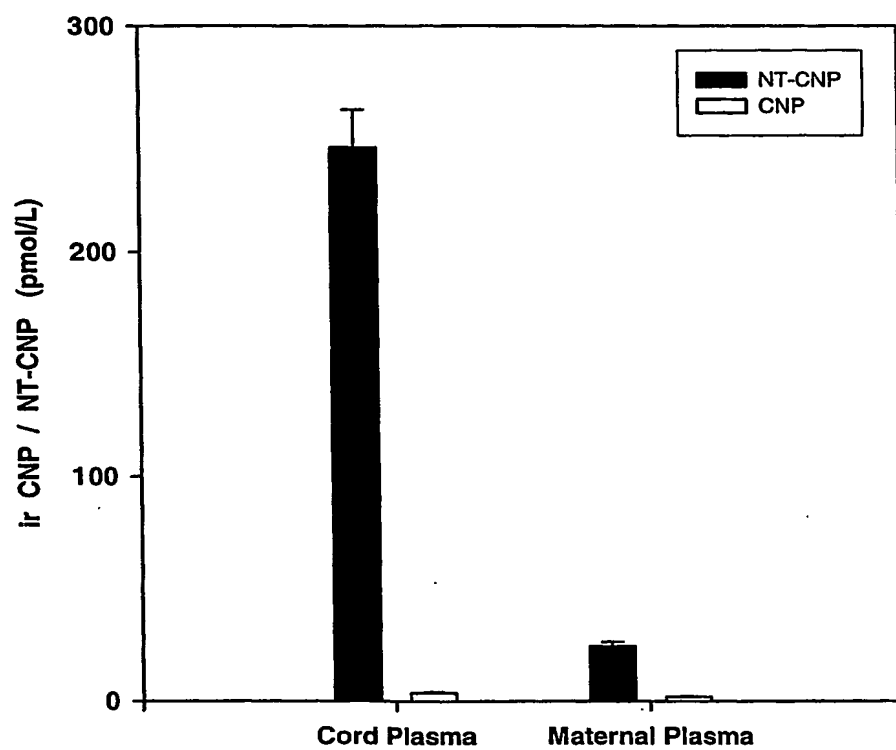


Figure 7

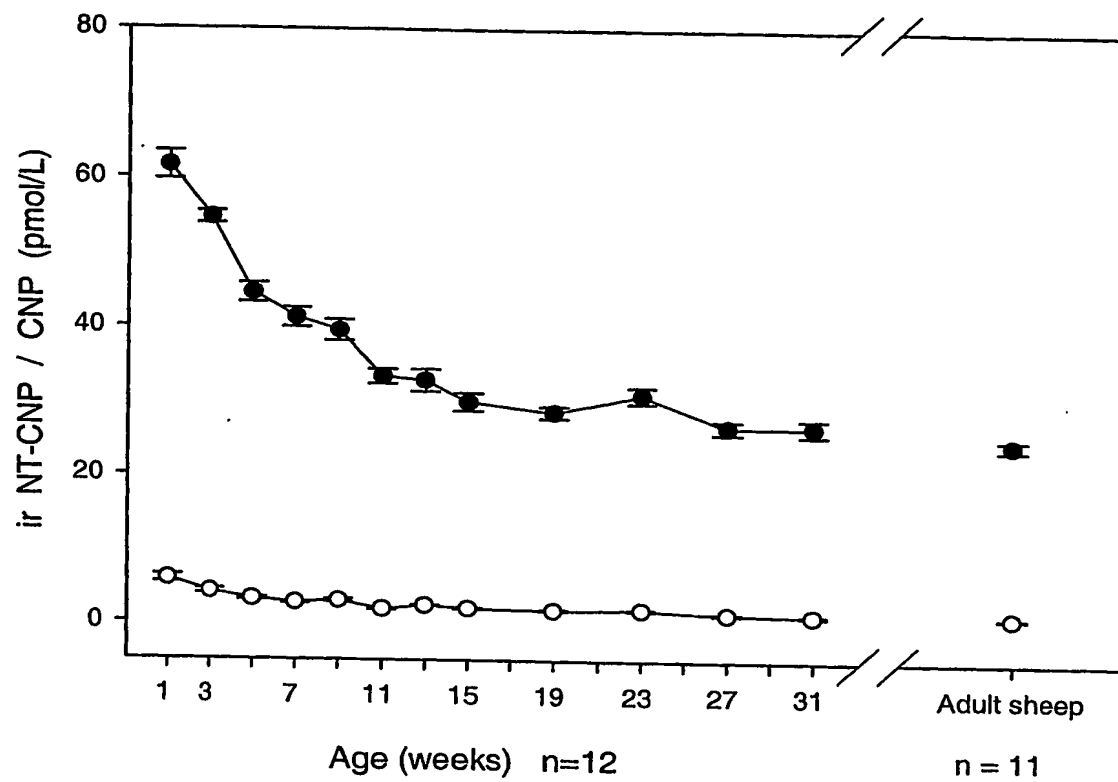
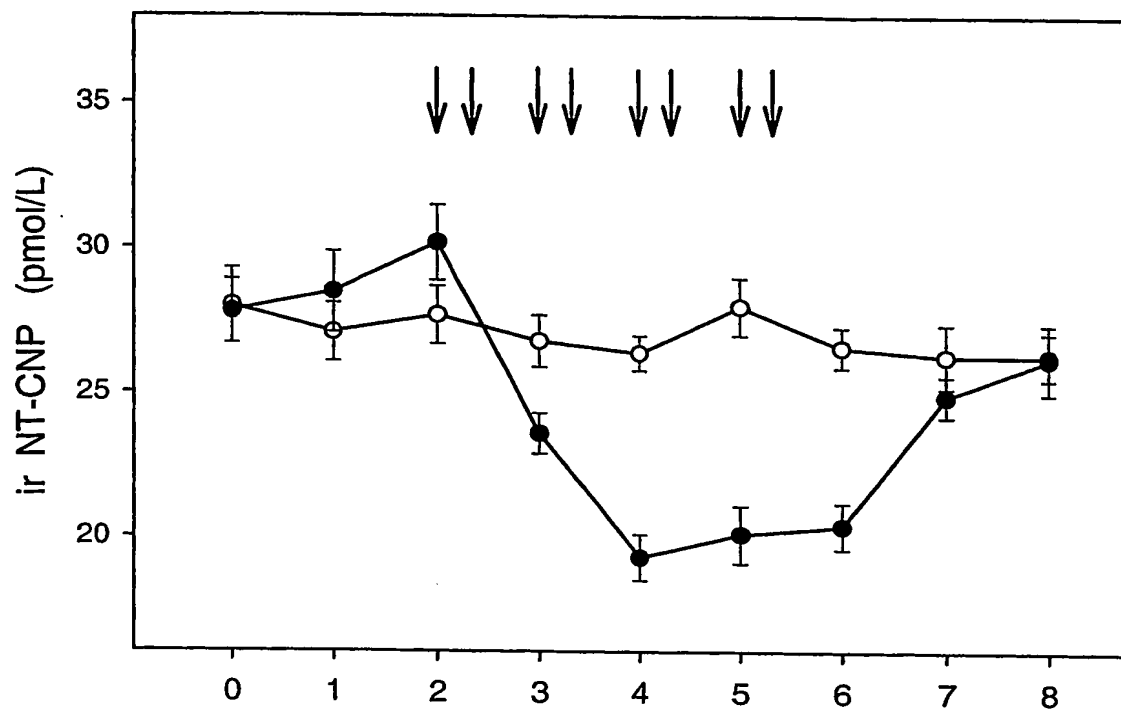


Figure 8

A



B

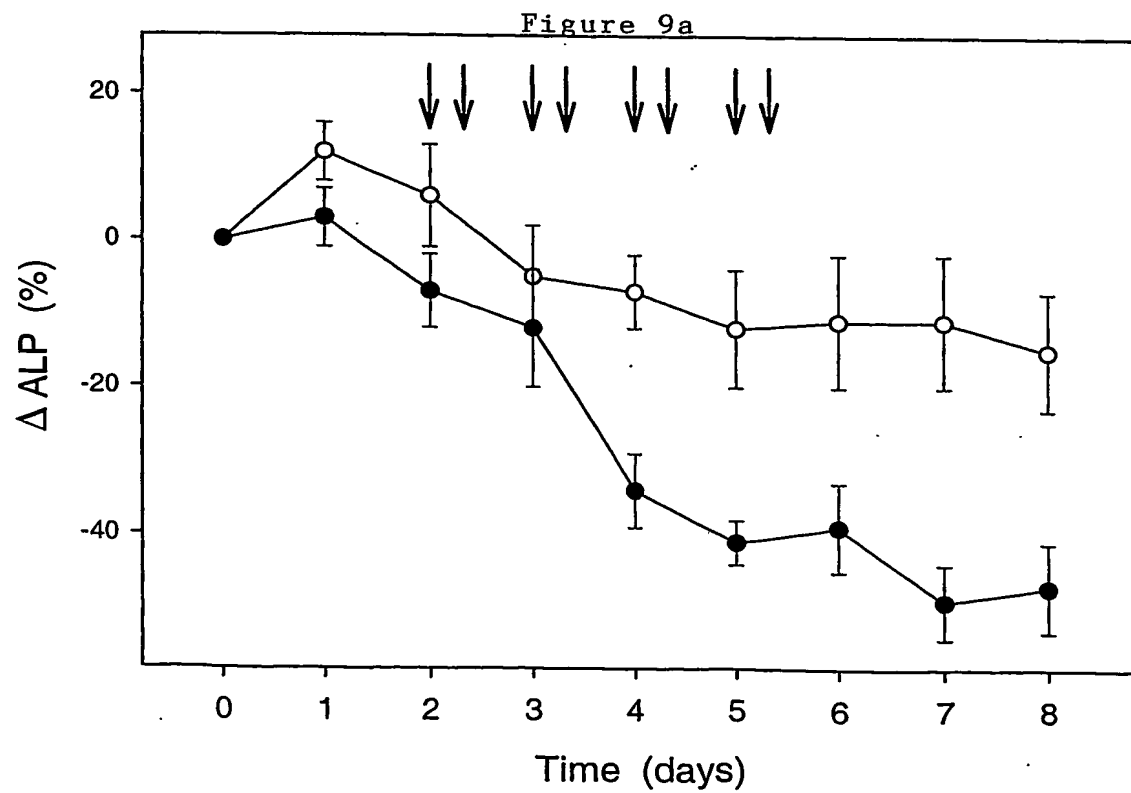


Figure 9b